



Comparative efficacy of North American and antigenically matched reverse genetics derived H5N9 DIVA marker vaccines against highly pathogenic Asian H5N1 avian influenza viruses in chickens

Samadhan J. Jadhao^a, Chang-Won Lee^{b,c}, Matt Sylte^a, David L. Suarez^{a,*}

^a Southeast Poultry Research Laboratory, Agricultural Research Service, United States Department of Agriculture, 934 College Station Road, Athens, GA 30605, USA

^b Food Animal Health Research Program, Ohio Agricultural Research and Development Center, The Ohio State University, Wooster, OH 44691, USA

^c Department of Veterinary Preventive Medicine, College of Veterinary Medicine, The Ohio State University, Columbus, OH 43210, USA

ARTICLE INFO

Article history:

Received 5 February 2009

Received in revised form 10 July 2009

Accepted 30 July 2009

Available online 15 August 2009

Keywords:

Avian influenza

Vaccine

H5N1

DIVA

Antigenic match

ABSTRACT

Highly pathogenic (HP) H5N1 avian influenza has become endemic in several countries in Asia and Africa, and vaccination is being widely used as a control tool. However, there is a need for efficacious vaccines preferably utilizing a DIVA (differentiate infected from vaccinated animals) marker strategy to allow for improved surveillance of influenza in vaccinated poultry. Using a reverse genetics approach, we generated Asian rgH5N9 vaccine strain deriving the hemagglutinin gene from A/chicken/Indonesia/7/2003 (H5N1) with modification of the cleavage site to be low pathogenic (LP) and N9 neuraminidase gene from the North American LP A/turkey/Wisconsin/1968 (H5N9) virus. The recombinant rgH5N9, A/turkey/Wisconsin/1968 (H5N9) A/chicken/Hidalgo/232/1994 (H5N2), and wild type HP A/chicken/Indonesia/7/2003 (H5N1) viruses were used to prepare inactivated oil-emulsified whole virus vaccines. Two weeks after vaccination, chickens were challenged with either Asian HP H5N1 viruses, A/chicken/Indonesia/7/2003 (W.H.O. clade 2.1) or A/chicken/Supranburi Thailand/2/2004 (W.H.O. clade 1.0). The H5 HA1 of the North American vaccine strains exhibited 12% amino acid differences including amino acid changes in the major antigenic sites as compared to the Asian HP H5N1 challenge viruses, serologically exhibited substantial antigenic difference, but still provided 100% protection from mortality. However, challenge virus shedding was significantly higher in chickens immunized with antigenically distinct American lineage vaccines as compared to the antigenically matched Asian rgH5N9 and the wild type Asian H5N1 vaccine. The antibody response to the heterologous subtype neuraminidase proteins were discriminated in vaccinated and infected chickens using a rapid fluorescent 2'-(4-methylumbelliferyl)- α -D-N-acetylneuraminic acid sodium salt as substrate for neuraminidase inhibition assay. This study demonstrates the value of using a vaccine containing antigenically matched H5 hemagglutinin for control of HP H5N1 avian influenza in poultry and the potential utility of a heterologous neuraminidase as a DIVA marker.

Published by Elsevier Ltd.

1. Introduction

Influenza A viruses have been isolated from many different avian and mammalian species, but wild waterfowl and shorebirds are thought to be the primary reservoir of influenza A virus in nature [1]. The influenza A virus genome consists of eight strands of negative sense RNA, which codes for 10–11 different proteins [2–5]. Influenza A viruses are characterized into distinct antigenic subtypes based on their two surface glycoproteins, the hemagglutinin (HA) and the neuraminidase (NA) [6]. Influenza A viruses representing 16 HA and 9 NA antigenic subtypes have been isolated

from avian species, and can theoretically be found in any combination of subtypes (e.g. H1N1, H5N9 etc.) [1,7]. The HA protein is initially synthesized as a single polypeptide precursor (HA0), which is then cleaved by host proteases into HA1 and HA2 subunits [8,9]. The clinical disease outcome in influenza A virus infection among avian species largely depends upon the ability of different cellular proteases to cleave the hemagglutinin protein. The presence of polybasic amino acids or an insert of amino acids at the HA cleavage site of H5 and H7 subtype viruses are characteristic of highly pathogenic (HP) viruses which by definition are highly lethal for chickens in standard animal pathotyping studies. Low pathogenic (LP) H5 and H7 viruses are distinctive in that they can quickly and unpredictably change to HP viruses with amino acid changes at the cleavage site [1,8]. In addition to the evolution of antigenic subtypes, avian influenza A viruses have evolved

* Corresponding author. Tel.: +1 706 546 3479; fax: +1 706 546 3161.

E-mail address: David.Suarez@ars.usda.gov (D.L. Suarez).

into distinct genetic lineages within the HA subtypes, Eurasian and North American lineages, presumably as a result of the predominant north to south migration pattern of their waterfowl hosts [1,11].

Highly pathogenic H5N1 avian influenza viruses cause a serious disease in poultry and is a notifiable disease to the Office International des Epizootics, the World Organization of Animal Health, and therefore outbreaks adversely affect international trade of poultry and poultry products [9]. An Asian lineage HP H5N1 avian influenza strain that emerged in domestic geese in southern China during 1996 [10] became endemic in the region, and continued to spread to other parts of the country and across Asia [11,12]. Beginning in late 2003, an unprecedented number of outbreaks of this H5N1 lineage occurred in many Asian, African and European countries [13,14]. Recent W.H.O. antigenic and phylogenetic analyses criteria distinguish Asian lineage H5N1 viruses into nine major clades, indicating the array of genetic and antigenic diversity among this lineage of avian influenza viruses, underscoring a challenge posed to control H5N1 avian influenza in poultry [15]. The zoonotic transmission of the HP H5N1 avian influenza virus strains from poultry to humans in Asian and African countries resulted in fatal illnesses in 241 of 383 confirmed cases and continue to pose a serious threat to the public health [16]. Because of the repeated outbreaks of HP H5N1 avian influenza in poultry in Asia and Africa, and associated human infections [17–22], control of the disease in poultry has become a high priority. Traditional control strategies against HP H5N1 avian influenza outbreaks in poultry include the identification and slaughter of affected and at risk poultry [23]. However, eradication by slaughter may be prohibitively costly when the outbreak is widespread within a country or region. Vaccination as a control tool has been gaining favor as a potentially more cost effective approach for controlling the virus, reducing the economic loss to poultry farmers, and contributing to improved food security in developing nations. Although several different types of vaccines are available worldwide, vaccination of poultry is primarily done with whole virus inactivated oil-emulsified vaccines that when properly administered can provide high antibody levels to the HA protein and result in significant reductions of viral shedding. Vaccination has been useful for the control of HP H5N1 avian influenza in chickens in Hong Kong [24] and H5N2 avian influenza in Mexico, although vaccination did not eradicate the LP H5N2 virus in Mexico [25].

One disadvantage of the inactivated whole virus vaccines is they affect serologic surveillance when using the common influenza type A specific diagnostic tests like the agar gel immunodiffusion test (AGID) or the commercially available enzyme linked immunosorbent assay, because both vaccinated and naturally infected birds develop antibody to both the nucleoprotein and matrix proteins which are the primary antigens for these tests. Ideally any vaccine approach would benefit from a DIVA (differentiate infected from vaccinated animals) strategy where an easy and cost effective serologic test can provide surveillance even in vaccinated flocks [23,26]. One proposed DIVA strategy is the heterologous neuraminidase (hNA) approach where the hemagglutinin subtype of the vaccine is matched to the predominantly circulating field strain, but the neuraminidase subtype is different from the field strain. Therefore the presence of antibodies or lack of antibodies to the neuraminidase protein can determine if a bird was infected, vaccinated or vaccinated and then infected with avian influenza virus. Inclusion of a rarely circulating NA subtype in poultry influenza virus such as N4, N5, N6, N8 and N9 in the vaccine, either a naturally occurring strain or in an engineered vaccine strain, can provide a useful and effective DIVA marker, and reduces the possibility of confusion if more than one subtype of avian influenza is circulating at one time [27,28]. The European Union has approved use

Table 1
Antigenic relatedness (cross-HI antibody titers) of North American and Asian H5 avian influenza A viruses used in vaccine and challenge experiments.

Immune anti-serum ^a	Viruses used as antigen ^b				
	A/turkey/Wisconsin/1968 (H5N9)	A/chicken/Hidalgo/232/1994 (H5N2)	A/chicken/Indonesia/7/2003 (rgH5N9)	A/chicken/Indonesia/7/2003 (wtH5N1)	A/chicken/Supranburi Thailand/2/2004 (wtH5N1)
A/turkey/Wisconsin/1968 (H5N9)	256^c	128	32	32	32
A/chicken/Hidalgo/232/1994 (H5N2)	64	128	32	32	16
A/chicken/Indonesia/7/2003 (rgH5N9)	32	32	256	256	128
A/chicken/Indonesia/7/2003 (wtH5N1)	32	16	128	128	128
A/chicken/Supranburi Thailand/2/2004 (wtH5N1)	32	32	128	128	256

wt: wild type virus; rg: reverse genetics derived mutated LPAI hemagglutinin cleavage site amino acid sequence RETR/GLF containing virus.

^a Immune anti-serum was produced by single dose s/c vaccination of 2-week-old SPF white Leghorn chickens with 0.5 ml of oil-emulsion vaccine. The serum samples from individual chickens were collected 3 weeks after immunization. Cross-HI assay was performed using immune serum from a single bird against each virus strain.

^b Influenza virus was inactivated using β-propiolactone and used as antigen in the micro-titer HI assay. Four HA units of viral antigen was used in the HI assay.

^c Endpoint HI titers with homologous virus strain (bold values) and heterologous H5 virus strains (non-bold values) in two-fold diluted serum samples.

Table 2

Serum HI antibody response of single dose NA DIVA marker vaccines and efficacy against A/chicken/Indonesia/7/2003 (W.H.O. clade 2.1) HP H5N1 virus challenge in chickens (experiment 1).

Vaccine	14 days post vaccination						10 days post-challenge						
	Log ₂ HI titers (SD) ^a			NA DIVA ^b			Birds shedding virus ^c		Log ₂ HI titers (SD) ^a	NA DIVA ^b			
	Vaccine Ag	Indo/03 Ag	Thai/04 Ag	N1	N2	N9	2 dpi	5 dpi	Vaccine Ag		N1	N2	N9
H5N9	6.6 (0.7)	3.7 (0.4) ^d	2.8 (0.8) ^d	0/8	0/8	8/8	8/8	8/8	9.0 (0.4)		8/8	0/8	8/8
H5N2	4.8 (0.8)	2.8 (1.3) ^d	2.6 (0.5) ^d	0/8	8/8	0/8	8/8	8/8	7.0 (0.6)		8/8	8/8	0/8
wtH5N1	5.2 (0.8)	5.2 (0.8)	4.5 (1.0)	8/8	0/8	0/8	3/8	2/8	6.5 (0.7)		8/8	0/8	0/8
rgH5N9/WSN	5.0 (1.0)	5.1 (1.3)	4.3 (1.0)	0/8	0/8	8/8	3/8	2/8	6.7 (0.4)		8/8	0/8	8/8
Control	<1.0	<1.0	<1.0	0/8	0/8	0/8	8/8	NA	NA		NA	NA	NA

wt: wild type virus; rg: reverse genetics derived mutated LPAI hemagglutinin cleavage site amino acid sequence RETR/GLF containing virus.

^a HI titers were measured in individual (unpooled) serum samples for each bird in the vaccinated groups collected 2-week post-vaccination and 10 days post-challenge, and the titers are expressed as mean (standard deviation) log₂ reciprocal of the endpoint in two-fold serum dilution for each group.

^b NA DIVA test were performed with N1, N2 and N9 antigens with serum samples collected 2-week post vaccination from vaccinated groups and 10-day post-challenge in the vaccinated and challenged groups. Vaccine Ag: homologous antigen used in HI assay, Indo/2003 Ag: A/chicken/Indonesia/7/2003 (H5N1) wt virus antigen, Thai/04 Ag: A/chicken/Supranburi Thailand/2/2004 (H5N1) wt virus antigen.

^c Detection of virus shedding from oropharyngeal swabs collected at 2 and 5 days post-challenge, number of birds virus positive/number of birds tested.

^d Kruskal–Wallis nonparametric analysis and Dunn's multiple comparison test indicates significantly ($P < 0.01$) reduced cross-HI reactivity of the serum collected from chickens vaccinated with North American H5 vaccine strains (H5N9, H5N2) and Eurasian W.H.O. clades 1.0 and 2.1 H5N1 virus strains used in this study.

Table 3

Serum HI antibody response and efficacy of single dose NA DIVA marker vaccines and efficacy against A/chicken/Supranburi Thailand/2/2004 (W.H.O. clade 1.0) HP H5N1 virus challenge in chickens (experiment 1).

Vaccine	14 days post vaccination						10 days post-challenge						
	Log ₂ HI titers (SD) ^a			NA DIVA ^b			Birds shedding virus ^c		Log ₂ HI titers (SD) ^a		NA DIVA ^b		
	Vaccine Ag	Indo/03 Ag	Thai/04 Ag	N1	N2	N9	2 dpi	5 dpi	Vaccine Ag		N1	N2	N9
H5N9	6.6 (0.7)	3.5 (0.7) ^d	3.0 (0.5) ^d	0/8	0/8	8/8	8/8	8/8	8.8 (0.6)		8/8	0/8	8/8
H5N2	5.0 (0.8)	2.3 (0.9) ^d	2.8 (0.3) ^d	0/8	8/8	0/8	8/8	8/8	7.0 (0.8)		8/8	8/8	0/8
wtH5N1	5.5 (1.3)	5.5 (1.3)	4.5 (1.0)	8/8	0/8	0/8	3/8	3/8	7.0 (0.9)		8/8	0/8	0/8
rgH5N9/WSN	5.5 (1.1)	5.6 (1.1)	4.5 (0.7)	0/8	0/8	8/8	2/8	2/8	7.2 (0.6)		8/8	0/8	8/8
Control	<1.0	<1.0	<1.0	0/8	0/8	0/8	8/8	NA	NA		NA	NA	NA

wt: wild type virus; rg: reverse genetics derived mutated LPAI hemagglutinin cleavage site amino acid sequence RETR/GLF containing virus.

^a HI titers were measured in individual (unpooled) serum samples for each bird in the vaccinated groups collected 2-week post-vaccination and 10 days post-challenge, and the titers are expressed as mean (standard deviation) log₂ reciprocal of the endpoint in two-fold serum dilution for each group.

^b NA DIVA test were performed with N1, N2 and N9 antigens with serum samples collected 2-week post vaccination from vaccinated groups and 10-day post-challenge in the vaccinated and challenged groups. Vaccine Ag: homologous antigen used in HI assay, Indo/2003 Ag: A/chicken/Indonesia/7/2003 (H5N1) wt virus antigen, Thai/04 Ag: A/chicken/Supranburi Thailand/2/2004 (H5N1) wt virus antigen.

^c Detection of virus shedding from oropharyngeal swabs collected at 2 and 5 days post-challenge, number of birds virus positive/number of birds tested.

^d Kruskal–Wallis nonparametric analysis and Dunn's multiple comparison test indicates significantly ($P < 0.01$) reduced cross-HI reactivity of the serum collected from chickens vaccinated with North American H5 vaccine strains (H5N9, H5N2) and Eurasian W.H.O. clades 1.0 and 2.1 H5N1 virus strains used in this study.

Table 4

Serum HI antibody response and efficacy of single dose NA DIVA marker vaccines against A/chicken/Indonesia/7/2003 (W.H.O. clade 2.1) HP H5N1 challenge virus in chickens (experiment 2).

Vaccine	14 days post vaccination					10 days post-challenge				
	Log ₂ HI titers (SD) ^a			NA DIVA ^b		Birds shedding virus ^d		Log ₂ HI titers (SD) ^a	NA DIVA ^b	
	Vaccine Ag	Indo/03 Ag	Thai/04 Ag	N1	N9	2 dpi	5 dpi	Vaccine Ag	N1	N9
H5N9	6.7 (0.8)	2.2 (1.0) ^e	2.7 (1.0) ^e	0/8	8/8	8/8	8/8	8.8 (0.6)	8/8	8/8
wtH5N1	5.8 (1.2)	5.8 (1.2)	4.1 (0.9)	8/8	0/8	3/8	2/8	7.2 (0.8)	8/8	0/8
wtH5N1 ^c	6.3 (0.9)	6.3 (0.9)	4.5 (0.7)	8/8	0/8	2/8	1/8	7.5 (0.5)	8/8	0/8
rgH5N9/WSN	4.8 (1.6)	4.7 (1.4)	4.3 (1.4)	0/8	8/8	3/8	2/8	6.5 (0.9)	8/8	8/8
rgH5N9/WSN ^c	5.6 (1.3)	5.6 (1.3)	4.1 (0.8)	0/8	8/8	2/8	1/8	7.5 (0.9)	8/8	8/8
Control	<1.0	<1.0	<1.0	0/8	0/8	8/8	NA	NA	NA	NA

wt: wild type virus; rg: reverse genetics derived mutated LPAI hemagglutinin cleavage site amino acid sequence RETR/GLF containing virus.

^a HI titers were measured in individual (unpooled) serum samples for each bird in the vaccinated groups collected 2-week post-vaccination and 10 days post-challenge, and the titers are expressed as mean (standard deviation) log₂ reciprocal of the endpoint in two-fold serum dilution for each group.

^b NA DIVA test were performed with N1, N2 and N9 antigens with serum samples collected 2-week post vaccination from vaccinated groups and 10-day post-challenge in the vaccinated and challenged groups. Vaccine Ag: homologous antigen used in HI assay, Indo/2003 Ag: A/chicken/Indonesia/7/2003 (H5N1) wt virus antigen, Thai/04 Ag: A/chicken/Supranburi Thailand/2/2004 (H5N1) wt virus antigen.

^c BPL inactivated influenza virus preparations were concentrated by ultracentrifugation pelleting (see Section 2) and the pellet was resuspended in reduced volumes of supernatant allantoic fluids to match the antigen titer with A/turkey/Wisconsin/1968 (H5N9) virus.

^d Detection of virus shedding from oropharyngeal swabs collected at 2 and 5 days post-challenge, number of birds virus positive/number of birds tested.

^e Kruskal–Wallis nonparametric analysis and Dunn's multiple comparison test indicates significantly ($P < 0.01$) reduced cross-HI reactivity of the serum collected from chickens vaccinated with North American H5 vaccine strains (H5N9, H5N2) and Eurasian W.H.O. clades 1.0 and 2.1 H5N1 virus strains used in this study.

Table 5
Serum HI antibody response and efficacy of single dose NA DIVA marker vaccines and efficacy against A/chicken/Supranburi Thailand/2/2004 (W.H.O. clade 1.0) HP H5N1 virus challenge in chickens (experiment 2).

Vaccine	14 days post vaccination					10 days post-challenge				
	Log ₂ HI titers (SD) ^a			NA DIVA ^b		Birds shedding virus ^d		Log ₂ HI titers (SD) ^a	NA DIVA ^b	
	Vaccine Ag	Indo/03 Ag	Thai/04 Ag	N1	N9	2 dpi	5 dpi	Vaccine Ag	N1	N9
H5N9	7.1 (1.2)	2.2 (1.1) ^e	2.1 (0.9) ^e	0/8	8/8	8/8	8/8	9.5 (0.9)	8/8	8/8
wtH5N1	5.7 (1.2)	5.7 (1.2)	4.3 (1.6)	8/8	0/8	3/8	3/8	7.3 (0.7)	8/8	0/8
wtH5N1 ^c	6.0 (0.9)	6.0 (0.9)	4.2 (0.4)	8/8	0/8	2/8	2/8	8.0 (0.8)	8/8	0/8
rgH5N9/WSN	4.8 (0.8)	4.6 (0.9)	4.1 (1.0)	0/8	8/8	2/8	0/8	6.9 (0.5)	8/8	8/8
rgH5N9/WSN ^c	5.5 (0.7)	5.3 (0.9)	4.7 (1.0)	0/8	8/8	1/8	0/8	7.0 (0.8)	8/8	8/8
Control	<1.0	<1.0	<1.0	0/8	0/8	8/8	NA	NA	NA	NA

wt: wild type virus; rg: reverse genetics derived mutated LPAI hemagglutinin cleavage site amino acid sequence RETR/GLF containing virus.

^a HI titers were measured in individual (unpooled) serum samples for each bird in the vaccinated groups collected 2-week post-vaccination and 10 days post-challenge, and the titers are expressed as mean (standard deviation) log₂ reciprocal of the endpoint in two-fold serum dilution for each group.

^b NA DIVA test were performed with N1, N2 and N9 antigens with serum samples collected 2-week post vaccination from vaccinated groups and 10-day post-challenge in the vaccinated and challenged groups. Vaccine Ag: homologous antigen used in HI assay, Indo/2003 Ag: A/chicken/Indonesia/7/2003 (H5N1) wt virus antigen, Thai/04 Ag: A/chicken/Supranburi Thailand/2/2004 (H5N1) wt virus antigen.

^c BPL inactivated influenza virus preparations were concentrated by ultracentrifugation pelleting (see Section 2) and the pellet was resuspended in reduced volumes of supernatant allantoic fluids to match the antigen titer with A/turkey/Wisconsin/1968 (H5N9) virus.

^d Detection of virus shedding from oropharyngeal swabs collected at 2 and 5 days post-challenge, number of birds virus positive/number of birds tested.

^e Kruskal–Wallis nonparametric analysis and Dunn's multiple comparison test indicates significantly ($P < 0.01$) reduced cross-HI reactivity of the serum collected from chickens vaccinated with North American H5 vaccine strains (H5N9, H5N2) and Eurasian W.H.O. clades 1.0 and 2.1 H5N1 virus strains used in this study.

of inactivated DIVA marker avian influenza vaccines for poultry in Italy [27]. Previously reported DIVA methods such as conventional NA inhibition (NI) assay [29] and cell culture based indirect fluorescent antibody test (FAT) [28] are not amenable for testing large numbers of samples in a timely way [9,28,30,31]. However, a recently described modification of the NI test using the fluorescent substrate 2'-(4-methylumbelliferyl)- α -D-N-acetylneuraminic acid sodium salt hydrate (MUN; Sigma Chemical Co., St. Louis, MO) [32], provides a fast, reliable and quantitative alternative [33].

Several commercial vaccines produced from LP Eurasian or North American H5 virus strains that naturally have a heterologous NA gene and allow for the potential of DIVA testing (N9, N3, N2) have been recently evaluated for their protective efficacy against Asian HP H5N1 viruses [34–37]. However, the HA amino acid sequences of these vaccine viruses are at times very divergent from that of circulating Asian HP H5N1 virus strains. The high sequence similarity between the HA protein amino acid sequence of the vaccine and challenge strain is known to be critical for optimal efficacy of the seasonal influenza vaccines in humans [38–40]. However, oil-emulsified poultry vaccines seem to provide much broader protection than human influenza vaccines, but a correlation in sequence similarity in regards to virus shedding after challenge has been observed [41]. An ideal vaccine against avian influenza should reduce shedding of the challenge virus to a minimum, and thus help reduce contamination of the environment and lateral spread of the virus [42–45]. A comparative study of the efficacy of inactivated H5 whole virus vaccines prepared from antigenically distinct North American and matched Asian H5 strains have not been performed against post 2003 Asian HP H5N1 avian influenza viruses in chickens. In this study, using a single vaccine dose, we report the comparative efficacy of inactivated oil-emulsified vaccines that were prepared from (i) naturally occurring antigenically distinct North American viruses [A/turkey/Wisconsin/1968 (H5N9) and A/chicken/Hidalgo/232/1994 (H5N2)] and (ii) reverse genetics based antigenically matched LP rgH5N9 virus derived from HP A/chicken/Indonesia/7/2003 (H5N1) virus strain (W.H.O. phylogenetic clade 2.1), against two HP Asian H5N1 avian influenza viruses belonging to the W.H.O. phylogenetic clade 1.0 and clade 2.1 with and without an engineered or naturally occurring hNA DIVA marker.

2. Materials and methods

2.1. Avian influenza viruses

The LP A/turkey/Wisconsin/1968 (H5N9), A/chicken/Hidalgo/232/1994 (H5N2), and HP A/chicken/Indonesia/7/2003 (H5N1) and A/chicken/Supranburi Thailand/2/2004 (H5N1) virus strains used in this study were obtained from the repository of the Southeast Poultry Research Laboratory. The viruses were diluted in sterile PBS supplemented with 1% (v/v) antibiotic and antimycotic solution (Cellgro, Manassas, VA) and passaged two times in 10-day-old specific pathogen free embryonated chicken eggs (SPF-ECE) via the chorioallantoic cavity route. The infected eggs were incubated at 37 °C for 3–4 days and then chilled at 4 °C. Allantoic fluid was harvested and virus titers were determined by hemagglutination assay using 0.5% (v/v) packed chicken erythrocytes. Aliquots of virus were stored at –70 °C for use in further experiments. Fifty percent egg infectious (EID₅₀) titers were determined by infecting eggs at log₁₀ limiting dilutions and titers were calculated by Reed and Muench method [46].

2.2. Molecular analysis of H5 hemagglutinin of vaccine and challenge viruses

Viral RNA was extracted from the vaccine seed and challenge virus stocks using viral RNA mini kit (Qiagen Inc., Valencia, CA). The H5 HA gene was amplified using a one step RT-PCR kit (Qiagen Inc., Valencia, CA) with gene segment specific primers that possessed flanking Bsm BI restriction enzyme sites. The direct nucleotide sequencing for HA gene was performed using PRISM Ready Reaction DyeDeoxy Terminator Cycle Sequencing kit (PerkinElmer, Foster City, CA) and was run on an automated sequencing 3730 PerkinElmer machine. Phylogenetic analysis of the HA1 sequences was performed by neighbor joining method using MEGA 3.1 version. Using a total of 1033 complete H5 A/goose/Guangdong/1996 (H5N1)-lineage HA nucleotide sequences available (avian, human and other mammalian isolates) in the NCBI GenBank for viruses isolated all over the world, two consensus H5 HA1 amino acid sequences were generated. The first consensus consist of viruses isolated from 1996 to 2004 and the second consensus for viruses isolated from 2005 to 2008. The H5 HA1 consensus and deduced

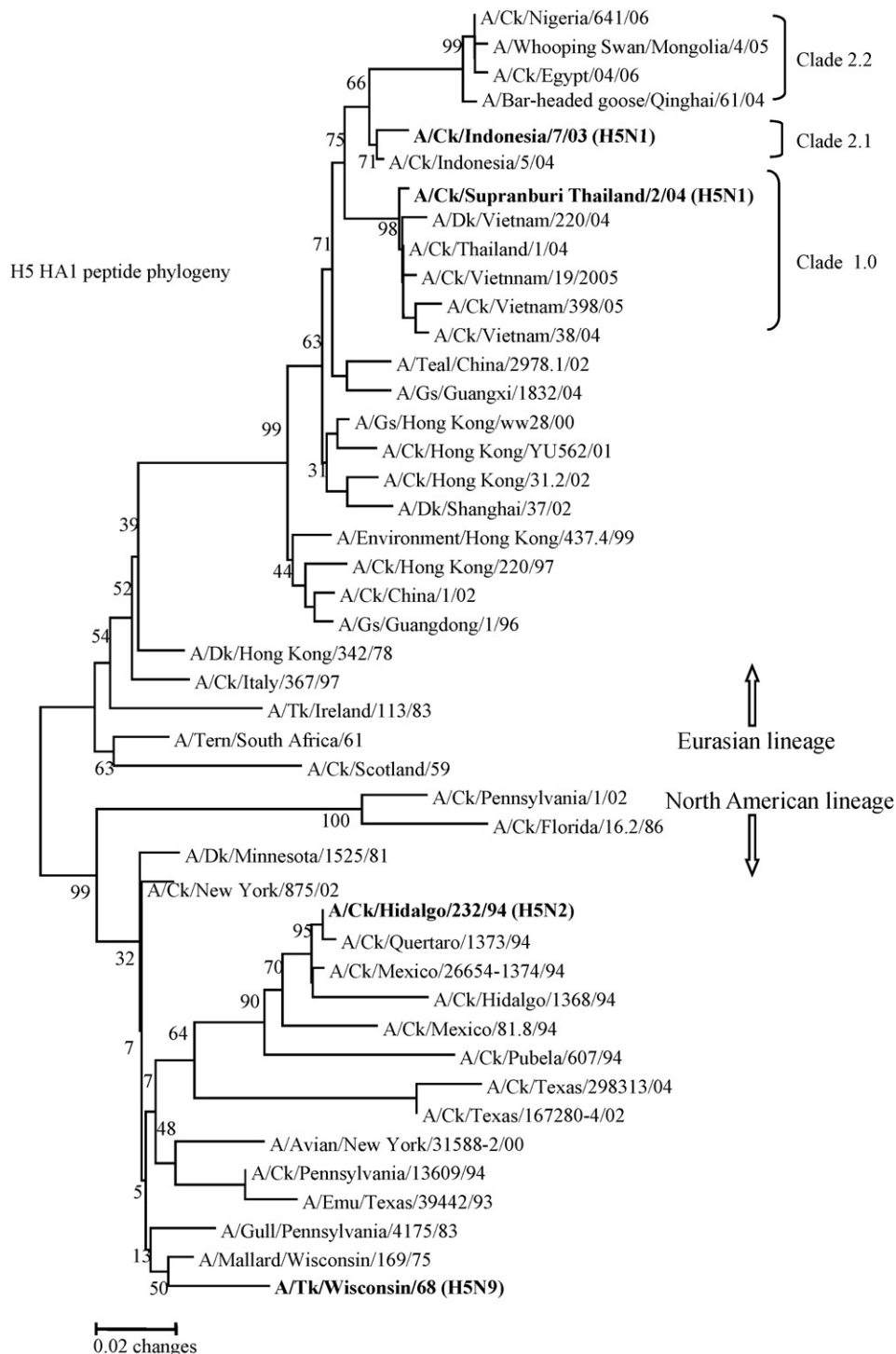
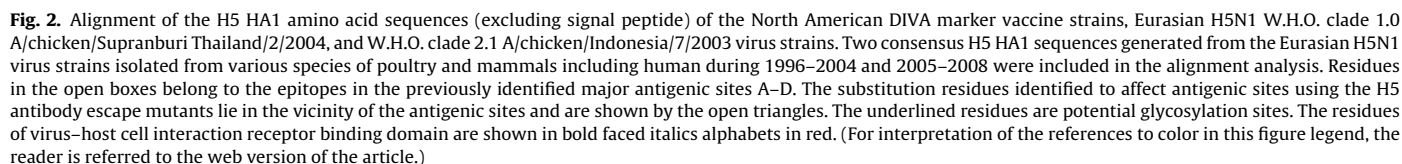


Fig. 1. Amino acid sequence based phylogenetic analysis of the North American neuraminidase DIVA marker H5 vaccine strains (H5N9 and H5N2) and Asian H5N1 challenge virus strain H5 hemagglutinin sequence. Viruses belonging to different hemagglutinin phylogenetic clades isolated during different time period and more recently isolated strains were included in the analysis. The H5 hemagglutinin gene of A/chicken/Indonesia/7/2003 (H5N1) W.H.O. clade 2.1 virus was used to generate a low pathogenic Asian rgH5N9 vaccine strain using reverse genetics. A/chicken/Supranburi Thailand/2/2004 (H5N1) W.H.O. clade 1.0 virus and A/chicken/Indonesia/7/2003 (H5N1) W.H.O. clade 2.1 viruses were used for challenge to evaluate the efficacy of the antigenically distinct North American (H5N9 and H5N2) vaccines and antigenically matched Eurasian rgH5N9 DIVA marker vaccines in chickens.

H5 HA1 amino acid sequences of the North American and Asian viruses used in this vaccine efficacy study, were aligned using clustalW algorithm (Lasergene Inc., Madison, WI). The amino acid sequence variation in the H5 HA1 antigenic sites and potential glycosylation motifs between the North American, and Asian viruses were identified and mapped in the H5 HA antigenic sites of A/duck/Singapore/3/1997 virus [47].

2.3. Generation of LP vaccine strain and safety test in chickens

The H5 HA gene segment amplicon of HP A/chicken/Indonesia/7/2003 (H5N1) virus was digested with *Bsm* BI restriction enzyme and cloned in the pHH21 reverse genetics vector. Using site directed mutagenesis, the HA1–HA2 cleavage site amino acid sequence RERRRKKR of HP A/chicken/Indonesia/7/2003 virus was



mutated to the LP cleavage site having amino acid sequence RETR. Recombinant virus containing the mutated LP H5 HA gene from A/chicken/Indonesia/7/2003 (H5N1) virus, N9 NA gene from American LP A/turkey/Wisconsin/1968 (H5N9) virus and internal genes from A/WSN/1933 (H1N1) virus was rescued in human embryonic kidney 293T cells, as described previously [28]. The pHH21 reverse genetics vector, reference transcription and expression plasmids were kindly provided by Dr. Yoshihiro Kawaoka, University of Madison, Wisconsin, USA [48]. The recombinant viruses were propagated in SPF embryonating chicken eggs. The viral RNA was extracted, the H5 HA gene was RT-PCR amplified and nucleotide sequencing was performed to confirm the mutated LP amino acid sequence RETR at the HA1–HA2 cleavage site of the recombinant rgH5N9 virus.

The pathogenicity and respiratory replication of the rgH5N9 marker vaccine strain was assessed by intranasal inoculation of 10^6 EID₅₀/0.2 ml virus in 4-week-old white Leghorn SPF chickens [49]. The chickens were examined routinely for clinical signs and mortality for 10 days post infection. Oropharyngeal and cloacal swabs were collected at 2 and 5 days post infection to assess the virus replication and shedding. Serum samples were collected at 10 days post infection to assess seroconversion.

2.4. Vaccines

The reverse genetics derived LP rgH5N9 marker vaccine strain, wild type North American A/turkey/Wisconsin/1968 (H5N9) vaccine strain, and wild type Asian A/chicken/Indonesia/7/2003 (H5N1) viruses were propagated at 37 °C for 2–4 days in the chorioallantoic sac of 9-day-old SPF-ECE. Allantoic fluid containing virus was clarified by low speed centrifugation ($1000 \times g$) for 15 min, and all the viruses had hemagglutination titers of 512 HA units. The viruses were inactivated with 0.5% β -propiolactone (BPL) (Sigma Chemical Co., St. Louis, MO) for 4 h at room temperature, transferred to 4 °C overnight for hydrolysis of the BPL and then the pH of the inactivated virus fluid was adjusted to 7.2 by the addition of sterile 7.5% sodium bicarbonate solution (w/v) [28]. Inactivation of the viruses was confirmed by their inability to replicate after inoculation in chorioallantoic sac of 9-day-old SPF-ECE [28]. Sterility of the inactivated viruses was confirmed by inoculating 0.1 ml inactivated antigen into sterile thioglycolate medium and incubation for 1 week at 37 °C.

The BPL inactivated virus antigens and oil phase were used to formulate water-in-oil emulsion adjuvant vaccines. The oil phase was formulated by mixing the pharmaceutical grade mineral oil Drakeol 6VR (Penreco, Butler, PA), Span 80 (Sigma Chemical Co., St. Louis, MO) and Tween 80 (Sigma Chemical Co., St. Louis, MO) at 36:3:1 ratio. Two days before vaccination, the oil phase and inactivated virus antigens were mixed at a ratio of 4:1 [28], homogenized in a Waring blender (Fisher Scientific International Inc., Hampton, NH) [50], and stored at 4 °C. The wild type H5N1 and rgH5N9 virus strains were concentrated by ultracentrifugation at $120,000 \times g$ for 2 h, and vaccines were prepared from concentrated virus preparations containing 1024 HA units/50 μ l. The North American A/chicken/Hidalgo/232/1994 virus based commercial Nobilis H5N2 vaccine (Intervet Inc.) was also used. The vaccines were equilibrated to room temperature on the day of vaccination, and mixed by vigorous shaking before administration to the chickens. Vaccine efficacy trial experiments were performed at two different times.

2.5. Protective efficacy of H5 NA DIVA marker vaccines

Four-week-old SPF white Leghorn chickens were obtained from the SEPRL hatchery and housed in a biosafety level 2 animal facility. The chickens were wing banded for identification, allowed to

acclimate for 2 days before vaccination and provided *ad libitum* access to food and water throughout the vaccination experiments. The vaccine efficacy was evaluated in chickens in two different experimental trials. In the first trial, 10 groups of eight birds in each group were used (Tables 2 and 3). In the second experimental trial, 12 groups of eight birds each were used (Tables 4 and 5). Pre-vaccination serum samples were obtained from the control groups in both experimental trials for use as negative samples in HI and heterologous subtype neuraminidase DIVA assays. The birds were vaccinated with 0.5 ml of vaccine by the subcutaneous route in the neck, and serologic antibody titers were determined 2 weeks after vaccination by the HI assay [9]. The vaccinated and control chickens were transferred to Horsfall units fitted with HEPA filter intake and exhaust and maintained under negative air pressure in a biosafety level 3 enhanced containment animal facility. The chickens were challenged by the oro-nasal route with 10^6 EID₅₀ dose (0.2 ml) of Asian H5N1 HP viruses, A/chicken/Supranburi Thailand/2/2004 (W.H.O. clade 1.0) or A/chicken/Indonesia/7/2003 (W.H.O. clade 2.1), respectively. At 10 days post-challenge, blood was collected and sera were harvested to evaluate the antibody response by HI assay. Oropharyngeal virus shedding titers were determined by inoculation of 9–11-day-old SPF-ECE with log₁₀ diluted oropharyngeal swab fluids [46].

2.6. Antigenic analysis and serum antibody response evaluation by HI assay

Antigenic analysis of the vaccine and challenge viruses was done by hemagglutination inhibition (HI) assay (Table 1) using anti-serum produced in SPF chickens 3 weeks after immunization with a single dose of β -propiolactone inactivated water-in-oil emulsified vaccines [42]. For antigenic analysis by cross-HI assay, immune serum from a single bird against the vaccine strains and challenge strains was used. To evaluate antibody response against the vaccines, individual (unpooled) serum samples from each immunized chickens in the groups were diluted two-fold in micro-titer plates using PBS, 4 HA units of inactivated virus antigens were added to the diluted sera and incubated at room temperature for 1 h to allow antigen–antibody interaction. Chicken erythrocytes (0.5%, v/v) were added to the wells in the assay according to W.H.O. protocol and serum dilution causing inhibition of the virus induced hemagglutination were considered endpoint titers [9]. Antigenic analysis and serum antibody response to H5 hemagglutinin of the vaccine was evaluated using homologous and heterologous H5 virus strains. The log₂ transformed values of the HI titers within and between groups were analyzed for statistical significance, and mean HI values with standard deviation for each group are presented in Tables 2–5.

2.7. Neuraminidase inhibition DIVA assay

The inhibition of influenza A virus vaccine subtype specific NA enzymatic activity by serum antibodies produced against the vaccine NA subtypes (N2 and N9) or the N1 NA subtype present in the challenge virus was performed as described previously [33] using the fluorescent substrate 2'-(4-methylumbelliferyl)- α -D-N-acetylneuraminic acid sodium salt hydrate (Sigma Chemical Co., St. Louis, MO) [32]. The NA DIVA test required the ability to differentiate the antibody response against NA subtype in the marker vaccine (N2 and N9) from that of the N1 NA of the challenge H5N1 virus. The extent of antigenic cross-reactivity between N2 and N9 NA subtypes of the vaccine viruses and the N1 NA subtype of the challenge virus was determined using 2-week post vaccination serum samples from both virus challenge groups/vaccine groups. The NA DIVA testing was performed in duplicate using two-fold diluted serum samples from individually vaccinated birds before and after challenge. The endpoint NI titers were determined as the preced-

ing log₂ serum dilution at which the fluorescence level begun to fall to the levels similar to the unvaccinated SPF chicken serum (pre-vaccination serum). The serum samples exhibiting ≥ 3 log₂ NI activity were considered positive for DIVA antibody test. The hNA DIVA test could not be applied to A/chicken/Indonesia/7/2003 (H5N1) vaccine group as the vaccine strain and Asian H5N1 challenge strain have the same subtype NA.

2.8. Statistical analysis

Serum antibody response and virus shedding data for vaccinated and control groups of chickens were analyzed using nonparametric Kruskal–Wallis test on GraphPad Prism v4.03 software package (GraphPad Software Inc., San Diego, CA). The statistical significance was set at $P < 0.05$. Dunn's multiple comparison was performed to all the groups in each experiment to assess the statistical significance.

3. Results

3.1. Molecular and antigenic analysis

Phylogenetic and sequence analysis of the H5 HA1 amino acid sequence alignment of the vaccine strains and

Asian challenge viruses is shown in Figs. 1 and 2. The H5 HA amino acid sequence based phylogeny indicates that North American A/turkey/Wisconsin/1968 (H5N9) and A/chicken/Hidalgo/232/1994 (H5N2) are distantly related to both Asian H5N1 lineage viruses A/chicken/Indonesia/7/2003 (H5N1) virus (W.H.O. clade 2.1) and A/chicken/Supranburi Thailand/2/2004 (W.H.O. clade 1.0). The H5 HA1 protein amino acid identity analysis revealed that the A/turkey/Wisconsin/1968 (H5N9) showed 11% divergence and A/chicken/Hidalgo/232/1994 (H5N2) vaccine strains showed 12% divergence from the Asian H5N1 viruses used for preparing antigenically matched vaccine or as a heterologous challenge virus (A/chicken/Supranburi Thailand/2/2004) in this study (Figs. 1 and 2). The H5 HA of North American A/turkey/Wisconsin/1968 virus showed 43 amino acid differences in the HA1 protein as compared to the Asian H5N1 challenge virus, A/chicken/Supranburi Thailand/2/2004. Of these amino acid variations, 14 were located in the antigenic sites previously identified for H1, H3 viruses and H5 antibody escape mutants (Figs. 2 and 3a). The Mexican vaccine strain had 46 amino acid differences in HA1, and 15 of those changes were located in putative antigenic sites (Figs. 2 and 3b) [47,51–57]. As compared to A/chicken/Indonesia/7/2003 virus, 45 amino acid changes were seen in HA1 protein of A/turkey/Wisconsin/1968

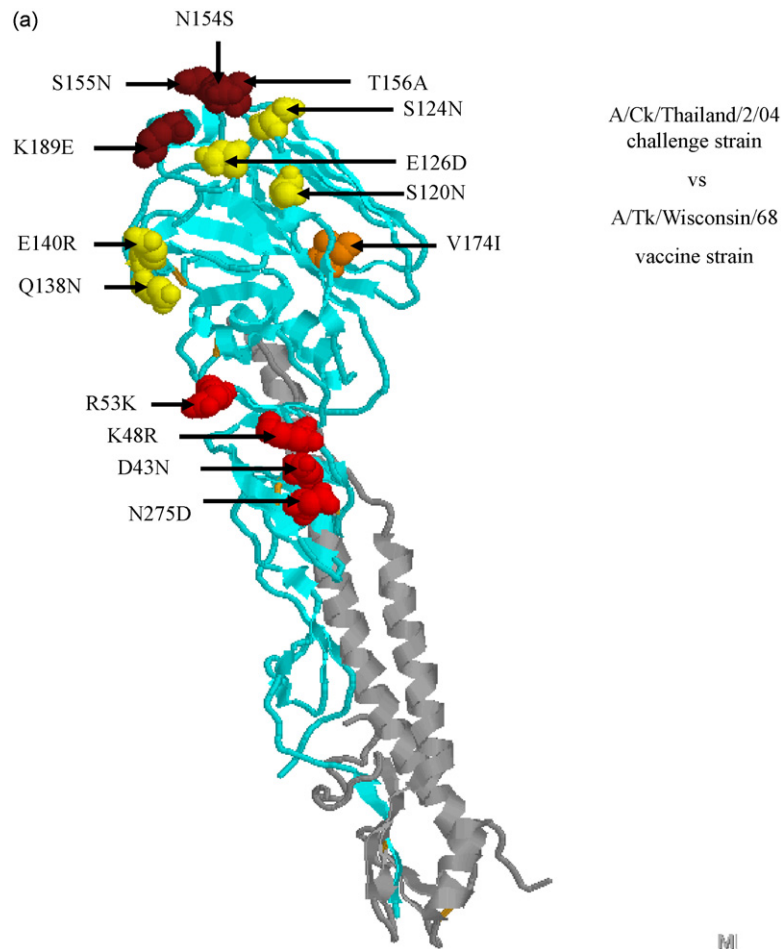


Fig. 3. The amino acid variation in the antigenic sites of the North American vaccine (A/turkey/Wisconsin/1968 H5N9 and A/chicken/Hidalgo/232/1994 H5N2) and Eurasian H5N1 A/chicken/Supranburi Thailand/2004 (W.H.O. clade 1.0) and A/chicken/Indonesia/7/2003 (W.H.O. clade 2.1) challenge virus strain HA1 sequences are depicted using the crystallized structure of the A/duck/Singapore/3/1997 virus H5 HA monomer. The color scheme yellow for antigenic site A, brown for antigenic site B, red for antigenic site C, orange for antigenic site D was used. (a) Amino acid variation in H5 HA antigenic sites between Eurasian A/chicken/Supranburi Thailand/2/2004 (H5N1) challenge and North American A/turkey/Wisconsin/1968 (H5N9) vaccine strains; (b) amino acid variation in H5 HA antigenic sites between Asian A/chicken/Supranburi Thailand/2/2004 (H5N1) challenge and North American A/chicken/Hidalgo/232/1994 (H5N2) vaccine strains; (c) amino acid variation in H5 HA antigenic sites between Asian A/chicken/Supranburi Thailand/2/2004 (H5N1) challenge strain and A/chicken/Indonesia/7/2003 (H5N1) strain used to generate a reverse genetics based marker rgH5N9 vaccine strain. Amino acid residue serine was not mutated at position 217. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

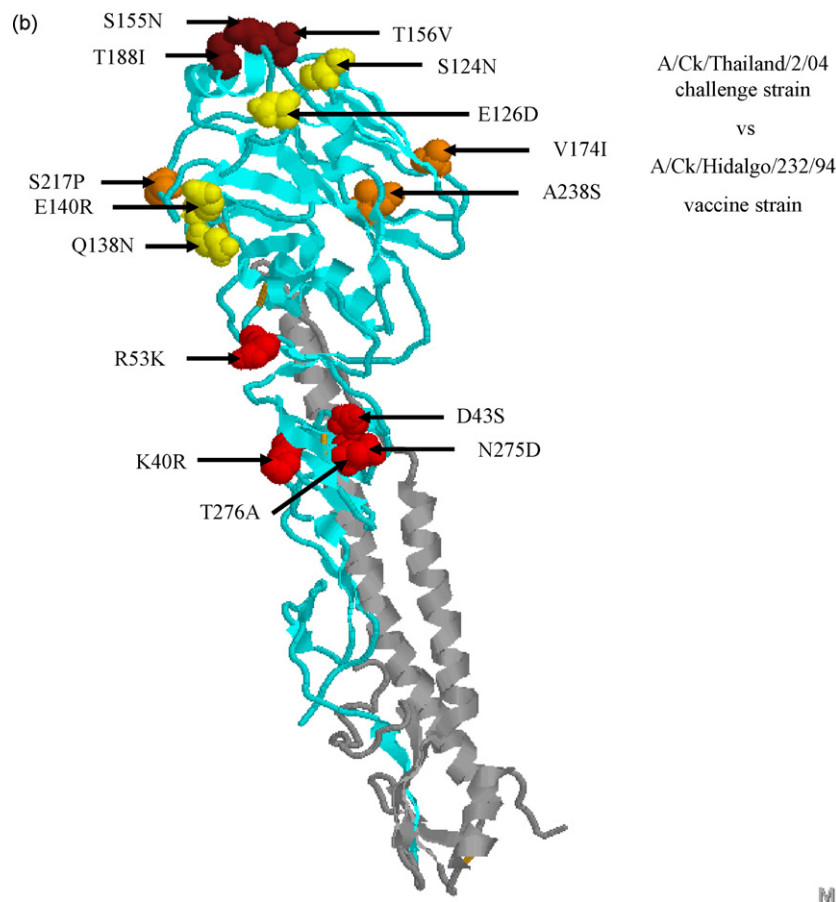


Fig. 3. (Continued)

virus whereas A/chicken/Hidalgo/232/1994 virus possessed 48 amino acid changes (Fig. 2). The HA1 amino acid sequence of A/turkey/Wisconsin/1968 (H5N9) vaccine strains differed from A/chicken/Hidalgo/232/1994 (H5N2) by 8% (26 of 326 amino acids) and of these 10 amino acid changes were located in antigenic sites (Fig. 2). The North American and A/chicken/Indonesia/7/2003 vaccine strains showed five potential glycosylation sites, but A/chicken/Supranburi Thailand/2/2004 had one less at position 154–156 in the antigenic site B. The amino acid residues in the receptor binding domains of the vaccine strains and Asian challenge strains were mostly conserved (Fig. 2), except a single variation each in 190 helix (T188I in North American A/chicken/Hidalgo/232/1994 vaccine strain) and R189E in North American A/turkey/Wisconsin/1968 vaccine strain) and 220 loop (S217P substitution in North American vaccine strains) regions of HA1 protein [58]. The HA1–HA2 cleavage site of the North American vaccine strains and reverse genetics derived Asian H5 mutant vaccine strain was characteristic (RETR) of wild type LP avian influenza viruses.

The antigenic analysis using immune serum produced against vaccine and challenge virus strains revealed that the North American vaccine strains are antigenically distinct from the Asian H5N1 viruses used in this study and exhibit a 4–8-fold HI titer difference (Table 1). The Asian A/chicken/Supranburi Thailand/2/2004 (W.H.O. clade 1.0) (H5N1) and A/chicken/Indonesia/7/2003 (H5N1) (W.H.O. clade 2.1) viruses showed two-fold differences in HI titer (Table 1). The HA1 of these two Asian H5N1 virus strains had only four amino acid variations, two of which were located in antigenic site B and remaining two changes were located in the sites pre-

viously identified using monoclonal antibody resistant H5 escape mutant strains (Figs. 2 and 3c).

The rgH5N9 (mutated low pathogenic cleavage site amino acid sequence RETR/GLF H5 HA from A/chicken/Indonesia/7/2003 H5, heterologous N9 subtype NA from A/turkey/Wisconsin/1968) virus was rescued and the attenuated phenotype was confirmed in a standard challenge of 10^6 EID₅₀ given *via* intranasal route to eight 4-week-old SPF WLH chickens. None of the birds showed any clinical signs of disease, no mortality, and no virus was detected from oropharyngeal swabs at 3 days post inoculation (dpi). All birds did seroconvert at 10 dpi when tested by hemagglutination inhibition assay, but with low titers.

Chickens administered a single dose of either antigenically distinct North American or antigenically matched rgH5N9 DIVA marker water-in-oil emulsion vaccine showed 100% protection against lethal challenge with both Asian H5N1 virus challenge strains, W.H.O. clade 1.0 A/chicken/Supranburi Thailand/2/2004 (H5N1) and W.H.O. clade 2.1 A/chicken/Indonesia/7/2003 (H5N1) viruses (Tables 2–5). Vaccinated chickens showed no signs of H5N1 clinical disease during the 10-day experiment. All of the unvaccinated control chickens succumbed to the Asian HP H5N1 viruses between 24–48 h after inoculation.

3.2. Antibody response following vaccination and challenge

Homologous and heterologous H5 virus strain HI antibody titers were assayed 2 weeks post vaccination, which preceded challenge with the Asian HP H5N1 virus strains (Tables 2–5). The BPL inactivated North American and Asian H5 virus antigens were used

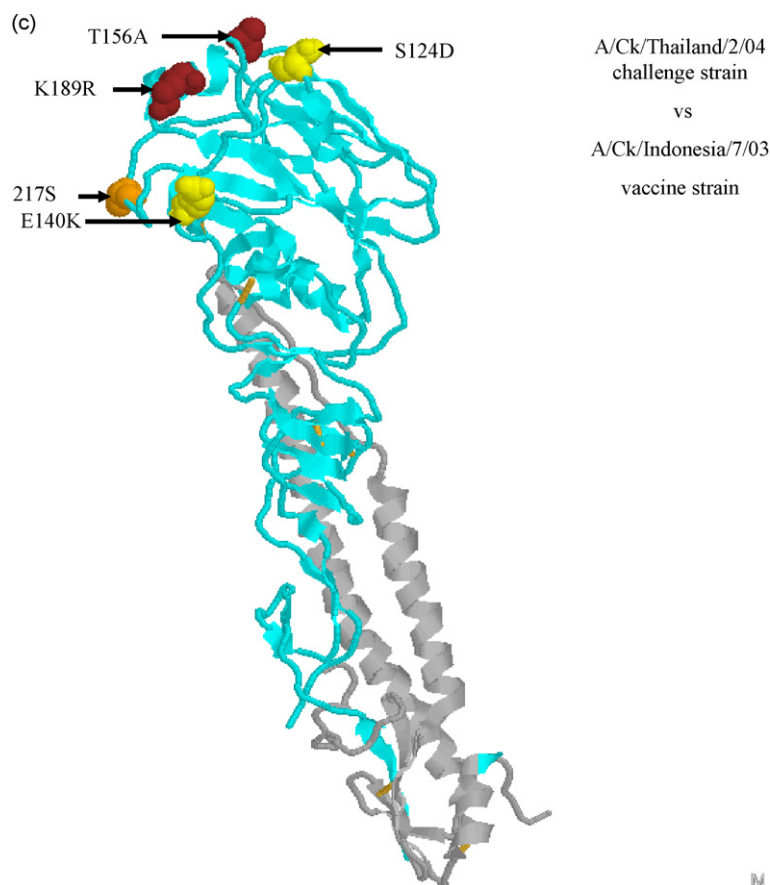


Fig. 3. (Continued).

in the HI assay. Prior to challenge, H5 HI antibodies were not detectable in the unvaccinated control group of chickens. The HI antibody titers in the range of 16–128 ($4-7 \log_2$) were detected in the chickens when the HI assay was done using homologous antigen (Tables 2–5). Use of the challenge strains as antigens was also included to assess cross-HI activity in serum of chickens vaccinated with North American H5N9 or H5N2 showed several fold reduced HI activity. Similarly reduced cross-HI activity was observed when North American H5 virus antigens were used to test serum samples from chickens that were vaccinated with Asian rgH5N9 marker vaccine. The serum titers in all the vaccinated groups increased by 10 days after challenge with the highest mean HI titer of 512 ($9 \log_2$) in A/turkey/Wisconsin/1968 (H5N9) group.

3.3. Challenge virus shedding

Oropharyngeal titers of the Asian HP H5N1 challenge viruses in unvaccinated control birds at necropsy, which died between 24–48 h post-challenge, ranged from 10^5 to 10^6 EID₅₀/ml (Fig. 4). As compared to the unvaccinated control groups, chickens vaccinated with North American vaccines showed a significantly lower levels of virus shedding ($P < 0.05$) on day 2 and day 5 post-challenge, but all the birds had detectable levels of virus shedding (Fig. 4). The North American vaccinated chickens had less virus shedding on day 5 as compared to day 2 post-challenge. In contrast a majority of the chickens vaccinated with antigenically matched Asian rgH5N9 marker vaccine did not shed challenge virus on day 2, and the birds that shed virus did so at low levels in the range of $1-2 \log_{10}$ EID₅₀/ml (Fig. 4). The level of virus shedding in the Asian rgH5N9 vaccine groups also decreased at 5 days post-challenge.

3.4. Neuraminidase inhibition DIVA assay

A quantitative NI assay was performed for serological differentiation of the vaccinated and infected chickens using MUN, a fluorescent NA substrate, and serum samples exhibiting $\geq 3 \log_2$ NI activities were considered positive [33]. In the vaccinated chickens, serum NI activity against the vaccine NA subtype was detectable 2 weeks after vaccination (Fig. 5). The unvaccinated chickens and chickens vaccinated with the N2 or N9 vaccines were below the cutoff for detectable N1 antibody before challenge. Ten days after the Asian H5N1 virus challenge of vaccinated groups of chickens, significantly ($P < 0.05$) higher levels of serum NI activity against the challenge virus N1 was observed in 100% of the birds, as compared to the serum from control group of chickens that were not exposed to influenza virus. During this period the NI titer against the NA subtype present in the vaccine (N9, N2) also continued to significantly rise likely because the birds were still responding to the primary vaccination. The hNA DIVA test could not be applied to A/chicken/Indonesia/7/2003 (H5N1) vaccine group as the vaccine strain and Asian H5N1 challenge strain have same subtype NA.

4. Discussion

Highly pathogenic H5N1 avian influenza has become endemic in several Asian and African countries and increasingly vaccination is being considered useful to prevent economic loss to poultry farmers and to better help control the disease. In poultry, inactivated oil-emulsified H5 avian influenza vaccines are still the most commonly used type of vaccine and the serum antibody response against HA glycoprotein plays a major role in protection by blocking

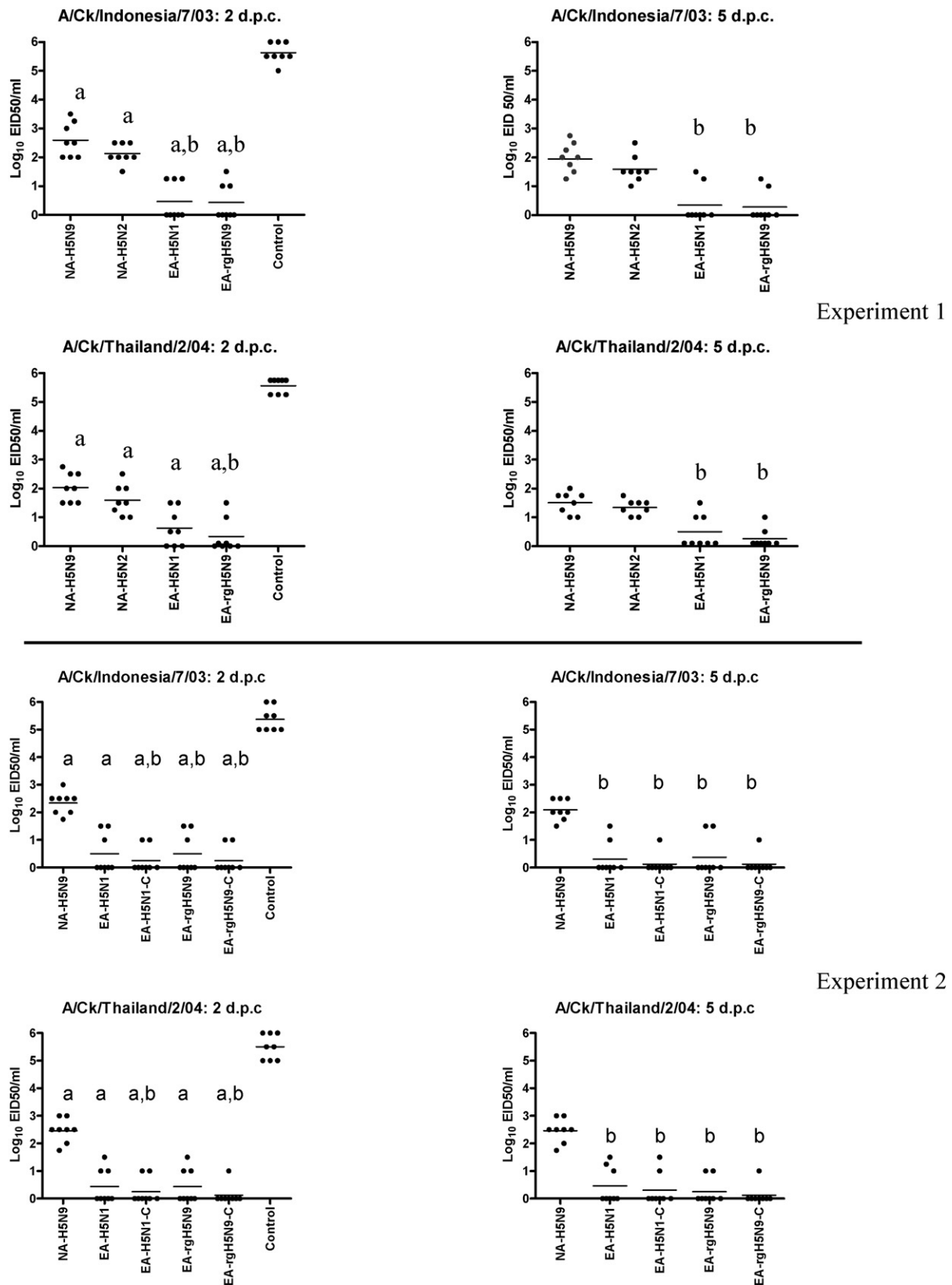


Fig. 4. Virus isolation (shedding) in embryonated chicken eggs from oropharyngeal swabs collected on day 2 and day 5 following challenge with highly pathogenic Eurasian H5N1 viruses, A/chicken/Indonesia/7/2003 (W.H.O. clade 2.1) and A/chicken/Supranburi Thailand/2/2004 (W.H.O. clade 1.0). Chickens in unvaccinated control groups showed 100% mortality between 24 and 48 h after challenge. The Kruskal–Wallis nonparametric statistical significance in virus shedding between groups is indicated by alphabets. a: statistically significant ($P < 0.05$) from unvaccinated control group; b: vaccines with antigenically matched H5 HA significantly ($P < 0.05$) reduce challenge virus shedding as compared to the North American A/turkey/Wisconsin/1968 (H5N9) vaccine.

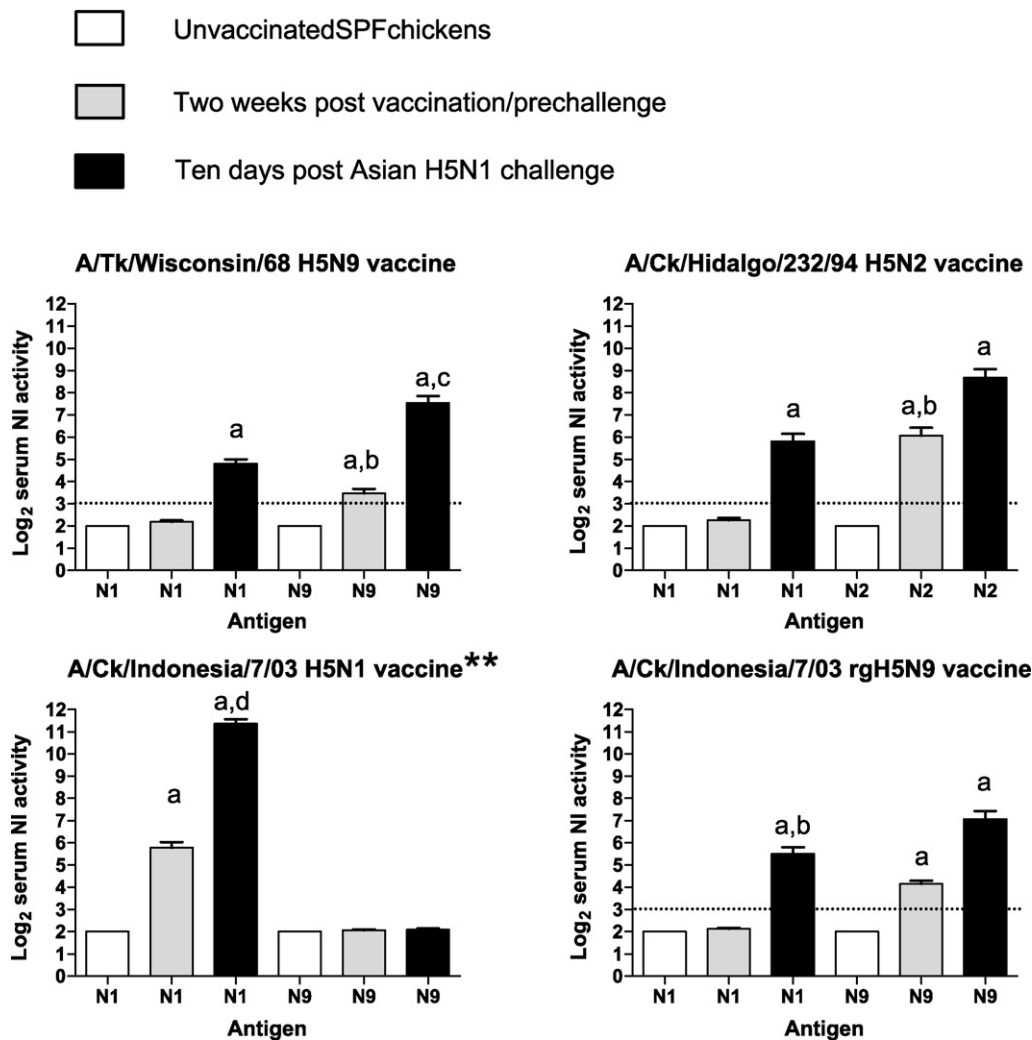


Fig. 5. Temporal serum neuraminidase inhibition activity against the heterologous subtype NA (N9, N2) of the DIVA marker vaccines and N1 NA of the Asian H5N1 challenge virus strains. Serum was collected from vaccinated and control chickens at 2 weeks after vaccination (pv). Vaccinated and control chicken groups were challenged with Eurasian H5N1 virus and serum was collected from surviving birds at 10 days post-challenge (pc). Unvaccinated control chickens died between 24 and 48 h pc. The levels of non specific NI activity in chickens which did not receive any viral antigen, and the extent of NI cross-reactivity of the DIVA marker N9 and N2 NA with that of the N1 NA of the Asian H5N1 challenge virus strains was plotted. The Kruskal–Wallis nonparametric statistical significance between groups is indicated by alphabets. a: significant difference in NI activity ($P < 0.05$) from control and cross-reaction with N1 NA; b: significant difference in NI activity ($P < 0.05$) from control and N1 NA pv; c: significant difference in NI activity ($P < 0.05$) from N1 NA pc; d: significant difference in NI activity ($P < 0.05$) from N9 NA pc. The number of sera used from vaccinated and challenged birds were, A/chicken/Indonesia/7/2003 H5N1 vaccine sera: $n = 31$, Asian rgH5N9 vaccine sera: $n = 55$, North American A/turkey/Wisconsin/1968 H5N9 vaccine sera: $n = 32$, North American A/chicken/Hidalgo/232/1994 H5N2 vaccine sera: $n = 16$, unvaccinated/pre-vaccination SPF chicken sera: $n = 16$. The serum samples from hNA subtype containing vaccine groups that exhibited $\geq 3 \log_2$ NI activity following HPAI H5N1 challenge (10 dpc) were considered positive for DIVA antibody test. Horizontal dotted line across the bars indicate positive cutoff NA DIVA test. EA: Eurasian lineage, NA: North American lineage. **: The hNA DIVA test could not be applied to A/chicken/Indonesia/7/2003 (H5N1) vaccine group as the vaccine strain and Asian H5N1 challenge strain has same subtype NA.

systemic replication of HP H5N1 virus and reducing virus shedding [23]. The success of vaccination for HP H5N1 avian influenza control program is influenced by the degree of antigenic match of the H5 HA protein in the vaccine strain. The first aim of the present study was to assess the influence of H5 antigenic variation of the vaccine strain on shedding of Asian H5N1 challenge virus. For this purpose, we compared protection conferred by antigenically distinct North American (H5N9 and H5N2) strain based vaccines, which are also commercially available, with antigenically matched Asian rgH5N9 experimental vaccine strain. The vaccinated chickens were challenged with two different Asian HP H5N1 viruses belonging to W.H.O. clades 1.0 and 2.1 (Fig. 1). The HA1 protein of the North American avian influenza A vaccine strains showed as much as 12% amino acid sequence divergence from both challenge viruses. We also used an early challenge model, challenge at 2 weeks after vaccination, to accentuate the differences between vaccines,

because peak levels of serum antibody usually occur 3–4 weeks post single vaccination. Evaluation of serum antibody response in vaccinated chickens at 2 weeks after immunization indicated that the inactivated H5 vaccines induced serum HI antibody response in the range of 16–128 ($4\text{--}7 \log_2$) against the homologous vaccine strain antigen. The HI antibodies titers were determined with homologous and heterologous antigen, and as expected the titers induced from the North American H5N9 and H5N2 vaccines exhibited much lower HI cross-reactivity (HI titers range of $2\text{--}3 \log_2$) with Asian H5N1 challenge virus antigens. Although the birds had lower specific antibody, the birds were still completely protected from morbidity and mortality. Similar observations were reported previously by performing Asian H5N1 HP virus challenge experiments using antigenically distinct vaccines [35,36]. The clinical protection seen with this low level of specific serum antibody is likely because of the effective blocking of systemic replication (viremia)

of the HPAI challenge virus preventing the systemic phase of virus replication that is usually required to cause death in chickens.

The data from the oropharyngeal virus shedding showed a much different result between vaccine groups. The North American H5 vaccinated chickens shed challenge virus at both time points, and they were shedding much higher levels of virus compared to the antigenically matched vaccinated chickens. The reduction in virus shedding is important for blocking the transmission of virus from one flock to another and also to reduce environmental contamination. These findings provide additional support for striving to match the H5 amino acid sequence identity of the vaccine with the challenge strain for optimal protection with oil-emulsified inactivated vaccines for poultry. Previous vaccine efficacy study in chickens using several North American H5 vaccine strains demonstrated that 40–100% of vaccinated chickens had oropharyngeal shedding of the HP North American A/chicken/Queretaro/14588/1995 challenge virus, depending on the degree of HA antigenic match between vaccine and challenge strains [59]. In another study, up to 10% mortality was reported in chickens receiving vaccines that were antigenically distinct from the highly pathogenic North American A/chicken/Queretaro/14588/1995 challenge virus [41].

Recent studies using two commercially available A/turkey/Wisconsin/1968 (H5N9) vaccines against A/chicken/Yamaguchi/7/2004 Asian HP H5N1 virus challenge indicated that even after two doses of vaccine there was oropharyngeal and cloacal virus shedding in 100% of the vaccinated chickens at day 3 and day 5 post-challenge [37]. Therefore, these large antigenic differences in the vaccine and circulating H5N1 virus strains is of concern if a primary goal of vaccination is to prevent spread of virus from vaccinated and infected flocks to the unvaccinated flocks. Therefore use of antigenically matched efficacious avian influenza vaccines could better serve as a barrier or a “ring” of vaccination around the quarantine zone [43].

To better understand the antigenic differences in HA protein and correlate with clinical cross protection and virus shedding, we mapped the H5 HA antigenic sites of the North American vaccine strains and Asian H5N1 virus strains. The analysis revealed presence of 14–15 amino acid changes in the predicted antigenic sites (A–E) [51,57], as well as amino acid residues that are known to be important for H5N1 escape mutant virus neutralization [53–57]. The H5 HA amino acid sequence differences between North American and the recent Asian H5N1 virus strains translate into the serological antigenic differences as seen in the cross-HI assay (Table 1). The serological cross-reactivity of the North American and Asian lineage viruses showed a 4–8-fold difference in HI titer. Selection of a new flu vaccine strain is usually done for seasonal human influenza vaccines if the vaccine strains in use exhibit more than four-fold antigenic difference in HI reactivity from the circulating strains [38–40]. Therefore, data from the present study suggests that the amino acid variations mapped in the antigenic sites (A–E) of the H5 virus together with the residues reported to contribute to neutralizing epitopes in the escape mutants are responsible for the observed antigenic differences.

The amino acid residues in the receptor binding domains (RBD) of the North American vaccine and Asian H5N1 challenge viruses were largely conserved. These conserved amino acid sequence in the 190 helix and 220 loop regions of HA1 protein RBD may also have contributed in the protection afforded by the North American H5N9 and H5N2 vaccines against the antigenically distinct Asian HP H5N1 challenge viruses. A recent study demonstrated contribution of the anti-H5 hemagglutinin RBD monoclonal antibodies in protection of mice and ferrets against HP H5N1 virus challenge, thus opening possibilities for allowing development of future H5 hemagglutinin RBD based vaccines [54]. The antigenic structure of the H5 HA is not completely elucidated and further analysis of

the neutralizing epitopes would help to understand the molecular and antigenic basis of the cross-reactivity and cross protection conferred by the antigenically distinct North American H5N9 and H5N2 vaccines against Asian HP H5N1 virus strains in chickens.

The second goal of this study was to evaluate the use of a hNA marker vaccine to differentiate vaccinated from vaccinated and infected animals (DIVA) that could prove beneficial for avian influenza surveillance programs in poultry [28,60,61]. However, most available techniques to detect serum influenza A virus NA antibody, such as the conventional neuraminidase inhibition assay [9,31] and indirect fluorescent antibody (IFA) methods are labor and time intensive [28,30]. Utility of a fluorescent MUN substrate based NI assay to quantitatively analyze the NA antibody response to the influenza NA vaccines in chickens has been demonstrated recently [33]. In this study, the assay was found to be a rapid and reliable way to distinguish between the N1, N2 and N9 NA subtype of the H5N1 challenge virus and marker vaccine strains. We successfully distinguished between vaccinated and infected birds as early as 10 days after challenge. These data emphasize the application of this assay with a possibility for higher throughput applications in avian influenza vaccine NA DIVA marker vaccine approach.

In summary, using reverse genetics technology we produced a vaccine strain with a matched hemagglutinin gene but with a different neuraminidase subtype to produce an efficacious vaccine that can be used as part of a DIVA surveillance program. Antigenically matching the vaccine to the challenge strain provided superior protection compared to poorly matched vaccines when virus shedding is used as the measure of protection. Finally, the use of MUN substrate to measure NA antibody titers appears to provide a more efficient method for testing than other conventional available methods.

Acknowledgements

This work was supported by U.S. Department of Agriculture (ARS CRIS project #6612-32000-048). We thank Suzanne DeBlois and Aniko Zsak for technical assistance. We acknowledge technical assistance of Joyce Bennett, SAA sequencing facility for automated sequencing. We thank Roger Brock for animal care assistance during the experiments.

References

- [1] Webster RG, Bean WJ, Gorman OT, Chambers TM, Kawaoka Y. Evolution and ecology of influenza A viruses. *Microbiol Rev* 1992;56(March (1)):152–79.
- [2] Palese P, Schulman JL. Mapping of the influenza virus genome: identification of the hemagglutinin and the neuraminidase genes. *Proc Natl Acad Sci USA* 1976;73(June (6)):2142–6.
- [3] Ritchey MB, Palese P, Schulman JL. Mapping of the influenza virus genome. III. Identification of genes coding for nucleoprotein, membrane protein, and nonstructural protein. *J Virol* 1976;20(October (1)):307–13.
- [4] Lamb RA, Choppin PW. Segment 8 of the influenza virus genome is unique in coding for two polypeptides. *Proc Natl Acad Sci USA* 1979;76(October (10)):4908–12.
- [5] Chen W, Calvo PA, Malide D, Gibbs J, Schubert U, Bacik I, et al. A novel influenza A virus mitochondrial protein that induces cell death. *Nat Med* 2001;7(December (12)):1306–12.
- [6] Johansson ME, Grandien M, Arro L. Preparation of sera for subtyping of influenza A viruses by immunofluorescence. *J Immunol Methods* 1979;27(3):263–72.
- [7] Fouchier RA, Munster V, Wallensten A, Bestebroer TM, Herfst S, Smith D, et al. Characterization of a novel influenza A virus hemagglutinin subtype (H16) obtained from black-headed gulls. *J Virol* 2005;79(March (5)):2814–22.
- [8] Alexander DJ. A review of avian influenza in different bird species. *Vet Microbiol* 2000;74(May (1–2)):3–13.
- [9] Webster RG, Cox N, Stohr K. WHO manual manual on Animal Influenza Diagnosis and surveillance. Available at: <http://www.who.int/emc-documents/influenza/doc/whocdscsrncs20025screen.pdf>; 2002.
- [10] Xu X, Subbarao, Cox NJ, Guo Y. Genetic characterization of the pathogenic influenza A/Goose/Guangdong/1/96 (H5N1) virus: similarity of its hemagglutinin gene to those of H5N1 viruses from the 1997 outbreaks in Hong Kong. *Virology* 1999;261(August (1)):15–9.

- [11] Guan Y, Peiris M, Kong KF, Dyrting KC, Ellis TM, Sit T, et al. H5N1 influenza viruses isolated from geese in Southeastern China: evidence for genetic reassortment and interspecies transmission to ducks. *Virology* 2002;292(January (1)):16–23.
- [12] Webster RG, Guan Y, Peiris M, Walker D, Krauss S, Zhou NN, et al. Characterization of H5N1 influenza viruses that continue to circulate in geese in southeastern China. *J Virol* 2002;76(January (1)):118–26.
- [13] The World Health Organization Global Influenza Program Surveillance Network. Evolution of H5N1 avian influenza viruses in Asia. *Emerg Infect Dis* 2005;11(October (10)):1515–21.
- [14] Li KS, Guan Y, Wang J, Smith GJ, Xu KM, Duan L, et al. Genesis of a highly pathogenic and potentially pandemic H5N1 influenza virus in eastern Asia. *Nature* 2004;430(July (6996)):209–13.
- [15] W.H.O. Towards a unified nomenclature system for the highly pathogenic H5N1 avian influenza viruses. http://www.who.int/csr/disease/avian_influenza/guidelines/nomenclature/en/2007.
- [16] W.H.O. Cumulative Number of Confirmed Human Cases of Avian Influenza A/(H5N1) Reported to WHO: http://www.who.int/csr/disease/avian_influenza/country/cases_table_2008_05_28/en/index.html; 2008.
- [17] Ducatez MF, Olinger CM, Owadga AA, Tarnagda Z, Tahita MC, Sow A, et al. Molecular and antigenic evolution and geographical spread of H5N1 highly pathogenic avian influenza viruses in western Africa. *J Gen Virol* 2007;88(August (Pt 8)):2297–306.
- [18] Lee CW, Suarez DL, Tumpey TM, Sung HW, Kwon YK, Lee YJ, et al. Characterization of highly pathogenic H5N1 avian influenza A viruses isolated from South Korea. *J Virol* 2005;79(March (6)):3692–702.
- [19] Ray K, Potdar VA, Cherian SS, Pawar SD, Jadhav SM, Waregaonkar SR, et al. Characterization of the complete genome of influenza A (H5N1) virus isolated during the 2006 outbreak in poultry in India. *Virus Genes* 2008;36(April (2)):345–53.
- [20] Rinder M, Lang V, Fuchs C, Hafner-Marx A, Bogner KH, Neubauer A, et al. Genetic evidence for multi-event imports of avian influenza virus A (H5N1) into Bavaria, Germany. *J Vet Diagn Invest* 2007;19(May (3)):279–82.
- [21] Terregino C, Milani A, Capua I, Marino AM, Cavaliere N. Highly pathogenic avian influenza H5N1 subtype in mute swans in Italy. *Vet Rec* 2006;158(April (14)):491.
- [22] Weber S, Harder T, Starick E, Beer M, Werner O, Hoffmann B, et al. Molecular analysis of highly pathogenic avian influenza virus of subtype H5N1 isolated from wild birds and mammals in northern Germany. *J Gen Virol* 2007;88(February (Pt 2)):554–8.
- [23] Suarez DL, Lee CW, Swayne DE. Avian influenza vaccination in North America: strategies and difficulties. *Dev Biol* 2006;124:117–24.
- [24] Ellis TM, Leung CY, Chow MK, Bisset LA, Wong W, Guan Y, et al. Vaccination of chickens against H5N1 avian influenza in the face of an outbreak interrupts virus transmission. *Avian Pathol* 2004;33(August (4)):405–12.
- [25] Villarreal-Chavez C, Rivera-Cruz E. An update on avian influenza in Mexico. *Avian Dis* 2003;47(Suppl. 3):1002–5.
- [26] Capua I, Cattoli G, Marangon S. DIVA—a vaccination strategy enabling the detection of field exposure to avian influenza. *Dev Biol* 2004;119:229–33.
- [27] Capua I, Terregino C, Cattoli G, Mutinelli F, Rodriguez JF. Development of a DIVA (differentiating infected from vaccinated animals) strategy using a vaccine containing a heterologous neuraminidase for the control of avian influenza. *Avian Pathol* 2003;32(February (1)):47–55.
- [28] Lee CW, Senne DA, Suarez DL. Generation of reassortant influenza vaccines by reverse genetics that allows utilization of a DIVA (differentiating infected from vaccinated animals) strategy for the control of avian influenza. *Vaccine* 2004;22(August (23–24)):3175–81.
- [29] Aymard-Henry M, Coleman MT, Dowdle WR, Laver WG, Schild GC, Webster RG. Influenzavirus neuraminidase and neuraminidase-inhibition test procedures. *Bull World Health Organ* 1973;48(2):199–202.
- [30] Cattoli G, Milani A, Bettini F, Serena Beato M, Mancini M, Terregino C, et al. Development and validation of an anti-N3 indirect immunofluorescent antibody test to be used as a companion diagnostic test in the framework of a “DIVA” vaccination strategy for avian influenza infections in poultry. *Avian Pathol* 2006;35(April (2)):154–9.
- [31] Van Deusen RA, Hinshaw VS, Senne DA, Pellacani D. Micro neuraminidase-inhibition assay for classification of influenza A virus neuraminidases. *Avian Dis* 1983;27(July–September (3)):745–50.
- [32] Potier M, Mameli L, Belisle M, Dallaire L, Melancon SB. Fluorometric assay of neuraminidase with a sodium (4-methylumbelliferyl- α -D-N-acetylneuraminate) substrate. *Anal Biochem* 1979;94(April (2)):287–96.
- [33] Sylte MJ, Hubby B, Suarez DL. Influenza neuraminidase antibodies provide partial protection for chickens against high pathogenic avian influenza infection. *Vaccine* 2007;25(May (19)):3763–72.
- [34] Beato MS, Toffan A, De Nardi R, Cristalli A, Terregino C, Cattoli G, et al. A conventional, inactivated oil emulsion vaccine suppresses shedding and prevents viral meat colonisation in commercial (Pekin) ducks challenged with HPAI H5N1. *Vaccine* 2007;25(May (20)):4064–72.
- [35] Bublot M, Le Gros FX, Nieddu D, Pritchard N, Mickle TR, Swayne DE. Efficacy of two H5N9-inactivated vaccines against challenge with a recent H5N1 highly pathogenic avian influenza isolate from a chicken in Thailand. *Avian Dis* 2007;51(March (Suppl. 1)):332–7.
- [36] Swayne DE, Lee CW, Spackman E. Inactivated North American and European H5N2 avian influenza virus vaccines protect chickens from Asian H5N1 high pathogenicity avian influenza virus. *Avian Pathol* 2006;35(April (2)):141–6.
- [37] Terregino C, Toffan A, Beato MS, De Nardi R, Drago A, Capua I. Conventional H5N9 vaccine suppresses shedding in specific-pathogen-free birds challenged with HPAI H5N1 A/chicken/Yamaguchi/7/2004. *Avian Dis* 2007;51(March (Suppl. 1)):495–7.
- [38] Smith DJ, Lapedes AS, de Jong JC, Bestebroer TM, Rimmelzwaan GF, Osterhaus AD, et al. Mapping the antigenic and genetic evolution of influenza virus. *Science* (New York, NY) 2004;305(July (5682)):371–6.
- [39] Russell CA, Jones TC, Barr IG, Cox NJ, Garten RJ, Gregory V, et al. Influenza vaccine strain selection and recent studies on the global migration of seasonal influenza viruses. *Vaccine* 2008;26(September (Suppl. 4)):D31–4.
- [40] Russell CA, Jones TC, Barr IG, Cox NJ, Garten RJ, Gregory V, et al. The global circulation of seasonal influenza A (H3N2) viruses. *Science* (New York, NY) 2008;320(April (5874)):340–6.
- [41] Swayne DE, Perdue ML, Beck JR, Garcia M, Suarez DL. Vaccines protect chickens against H5 highly pathogenic avian influenza in the face of genetic changes in field viruses over multiple years. *Vet Microbiol* 2000;74(May (1–2)):165–72.
- [42] Lee CW, Senne DA, Suarez DL. Effect of vaccine use in the evolution of Mexican lineage H5N2 avian influenza virus. *J Virol* 2004;78(August (15)):8372–81.
- [43] Suarez DL. Overview of avian influenza DIVA test strategies. *Biologicals* 2005;33(December (4)):221–6.
- [44] van der Goot JA, Koch G, de Jong MC, van Boven M. Quantification of the effect of vaccination on transmission of avian influenza (H7N7) in chickens. *Proc Natl Acad Sci USA* 2005;102(December (50)):18141–6.
- [45] van der Goot JA, van Boven M, Stegeman A, van de Water SG, de Jong MC, Koch G. Transmission of highly pathogenic avian influenza H5N1 virus in Pekin ducks is significantly reduced by a genetically distant H5N2 vaccine. *Virology* 2008;382(December (1)):91–7.
- [46] Reed LJ, Muench H. A simple method of estimating fifty percent endpoints. *Am J Hyg* 1938;27:493–7.
- [47] Ha Y, Stevens DJ, Skehel JJ, Wiley DC. H5 avian and H9 swine influenza virus haemagglutinin structures: possible origin of influenza subtypes. *EMBO J* 2002;21(March (5)):865–75.
- [48] Neumann G, Watanabe T, Ito H, Watanabe S, Goto H, Gao P, et al. Generation of influenza A viruses entirely from cloned cDNAs. *Proc Natl Acad Sci USA* 1999;96(August (16)):9345–50.
- [49] Subbarao K, Chen H, Swayne D, Mingay L, Fodor E, Brownlee G, et al. Evaluation of a genetically modified reassortant H5N1 influenza A virus vaccine candidate generated by plasmid-based reverse genetics. *Virology* 2003;305(January (1)):192–200.
- [50] Stone HD, Brugh M, Hopkins SR, Yoder HW, Beard CW. Preparation of inactivated oil-emulsion vaccines with avian viral or Mycoplasma antigens. *Avian Dis* 1978;22(October–December (4)):666–74.
- [51] Caton AJ, Brownlee GG, Yewdell JW, Gerhard W. The antigenic structure of the influenza virus A/PR/8/34 hemagglutinin (H1 subtype). *Cell* 1982;31(December (2 Pt 1)):417–27.
- [52] Ha Y, Stevens DJ, Skehel JJ, Wiley DC. X-ray structure of the hemagglutinin of a potential H3 avian progenitor of the 1968 Hong Kong pandemic influenza virus. *Virology* 2003;309(May (2)):209–18.
- [53] Kaverin NV, Rudneva IA, Govorkova EA, Timofeeva TA, Shilov AA, Kochergin-Nikitsky KS, et al. Epitope mapping of the hemagglutinin molecule of a highly pathogenic H5N1 influenza virus by using monoclonal antibodies. *J Virol* 2007;81(December (23)):12911–7.
- [54] Kaverin NV, Rudneva IA, Ilyushina NA, Varich NL, Lipatov AS, Smirnov YA, et al. Structure of antigenic sites on the haemagglutinin molecule of H5 avian influenza virus and phenotypic variation of escape mutants. *J Gen Virol* 2002;83(October (Pt 10)):2497–505.
- [55] Philpott M, Easterday BC, Hinshaw VS. Neutralizing epitopes of the H5 hemagglutinin from a virulent avian influenza virus and their relationship to pathogenicity. *J Virol* 1989;63(August (8)):3453–8.
- [56] Philpott M, Hioe C, Sheerar M, Hinshaw VS. Hemagglutinin mutations related to attenuation and altered cell tropism of a virulent avian influenza A virus. *J Virol* 1990;64(June (6)):2941–7.
- [57] Wiley DC, Wilson IA, Skehel JJ. Structural identification of the antibody-binding sites of Hong Kong influenza haemagglutinin and their involvement in antigenic variation. *Nature* 1981;289(January (5796)):373–8.
- [58] Yang ZY, Wei CJ, Kong WP, Wu L, Xu L, Smith DF, et al. Immunization by avian H5 influenza hemagglutinin mutants with altered receptor binding specificity. *Science* (New York, NY) 2007;317(August (5839)):825–8.
- [59] Swayne D, Beck JR, Garcia M, Stone HD. Influence of virus strain and antigen mass on efficacy of H5 avian influenza inactivated vaccines. *Avian Pathol* 1999;28:245–55.
- [60] Cattoli G, Terregino C, Brasola V, Rodriguez JF, Capua I. Development and preliminary validation of an ad hoc N1–N3 discriminatory test for the control of avian influenza in Italy. *Avian Dis* 2003;47(Suppl. 3):1060–2.
- [61] Liu M, Wood JM, Ellis T, Krauss S, Seiler P, Johnson C, et al. Preparation of a standardized, efficacious agricultural H5N3 vaccine by reverse genetics. *Virology* 2003;314(September (2)):580–90.